# Effect of rozmarinic acid supplementation on *in vitro* maturation of bovine oocytes

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***Abstract***

*Antioxidants supplementation of in vitro culture media exerts the key role to reduce the effects of reactive oxidative species produced during assisted reproduction technique. The objective of the study was to determine the effect of rosmarinic acid addition to the in vitro culture media on bovine oocytes maturation rate based on morphological changes. Bovine COC's were matured according to their morphological class (class I, II and III) in two groups: control (M) and supplemented with rosmarinic acid (105 µM, AR) in TCM 199 HEPES modification media at 38.5$^0$C in 5% $CO_2$ humidified air atmosphere for 24h. Comparing the groups, relative to the number of COC's matured, a increase in their maturation features is observed, with 26.81% % (AR1), 21.67% (AR2) and 23.34% (AR3), respectively in groups supplemented with rosmarinic acid. The oocyte class is associated with their capacity to develop in vitro based on their morphological examination.*

***Key words****: antioxidants, oocyte, rosmarinic acid*

## Introduction

*In vitro* fertilization (IVF) is an assisted reproduction technique (ART) used with good results in bovine reproduction, with 443.533 of bovine embryos obtained worldwide in the year 2012 according to statistics of the International Embrio Transfer Society (http://www.iets.org/pdf/comm_data/december2013.pdf)

Successful ART is influenced by many factors, among which reactive oxygen species (ROS) has a significant role (Agarwal et al., 2014).

Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Agarwal et al., 2014).

Reproductive systems possess antioxidant defense mechanisms that maintain equilibrium between pro- and anti-oxidants (Roychoudhury et al., 2017; Agarwal et al., 2014); but during *in vitro* conditions, the gametes needs to be protected by supplemented antioxidants.

Studies indicates that supplementing maturation media with different antioxidants such as β-mercaptoethanol (Sadeesh et al., 2014), cysteamine (Beheshti et al., 2011); cysteine (Mircu et al., 2015), vitamin C (Sovernigo et al., 2017; Comizzoli et al. 2003; Agarwal et al., 2014), plant antioxidants – flavonoids (Kang et al., 2016, Mbemya et al., 2017) can improve oocytes maturation based on nuclear morphological changes and on gene expression.

Another natural antioxidant used in ART, especially in freezing extenders were improves sperm quality after cryopreservation, is rosmarinic acid (Malo et al., 2010; Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid derived from hydroxycinnamic acid, that belongs to polyphenols group and is found as an active compound in several medicinal plants (*Rosmarinus officinalis, Salvia officinalis, Mentha arvense, Ocimum basilicum, Thymus vulgaris* etc) (Krajcovicova et al, 2013).

Rosmarinic acid has antiinflamatory, antiviral, antibacterial, antimutagen, antidepressant, antiallergic, antioxidant effects. His antioxidant activity is supporting by enhancement of superoxide and hydroxyl scavenging (Krajcovicova et al, 2013).

Hajhosseini et al. (2013) observed that rosmarinic acid has a preventive effect on Sertoli cells apoptosis caused by electromagnetic fields.

Although in literature are data regarding beneficial effects of green tea polyphenols (Wang et al., 2007; Ly et al., 2015) and catechins (Roychoudhury et al., 2017) on reproductive health and on IVF parameters and subsequent development, there are no studies, to our knowledge, regarding the effect of rosmarinic acid supplementation on bovine oocytes *in vitro* maturation. For this reason the purpose of this present research was to evaluate the effect of rozmarinic acid added in media for in vitro bovine oocytes maturation on their maturation rate based on morphological changes.

**Materials and methods**

Bovine ovaries (n=18) were collected from local slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep), at 33-35$^0$C within two hours. Handling medium for COC (cumulus -oocytes- complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep (17-602F, Lonza); 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4$^0$C): *I$^{st}$ class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *II$^{nd}$ class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III$^{d}$ class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium was prepared in our laboratory after Parrish et al. (1986) protocol with minor modifications: TCM 199 HEPES modification media, (M2520) with 10% ECS and 15 µl FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental group we added rosmarinic acid (105 µM)(536954, Sigma Aldrich) - *group AR*. Pools of 8-10 COCs were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5$^0$C in 5% $CO_2$ humidified air atmosphere for 24h. After 24h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed. The COC's were maturated according to there their morphological class (M1, M2, M3, AR1, AR2, AR3).

**Results and discussions**

The results of supplementation of *in vitro* media with rosmarinic acid on bovine oocytes morphological aspects are presented in figure 1 and 2.

After *in vitro* maturation of cow's oocytes in the medium without antioxidants (M group) we noticed at the morphological assessment that 55% of class I COCs (M1), 53.33% of class COC II (M2) and 26.66% of class III COCs (M3) were matured. In the rosmarinic acid supplemented groups (AR group), 81.81% of class I COCs (AR1) were matured after 24 hours, 75% of class II (AR2) and 50% of class III (AR3).

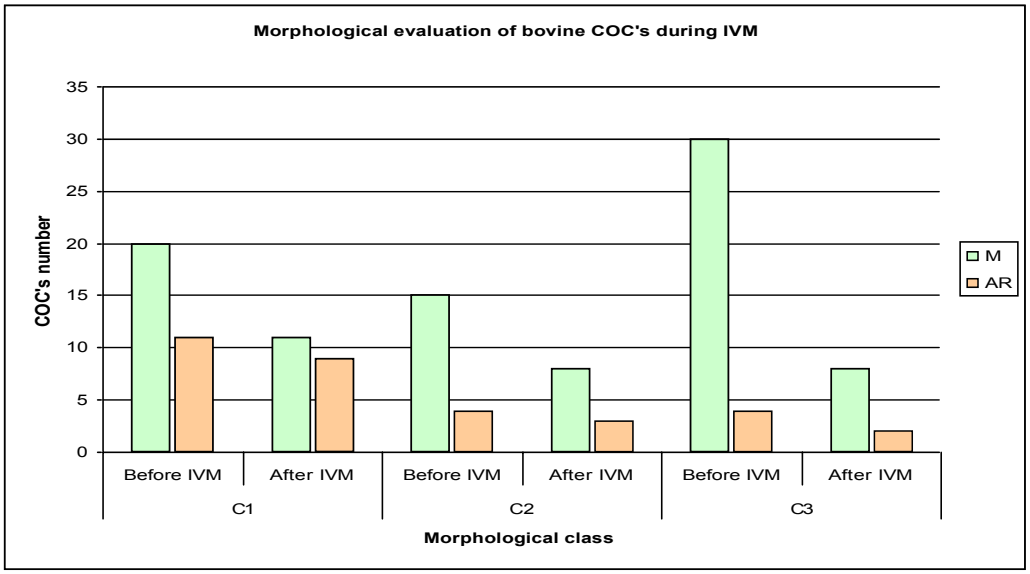


**Figure 1.** Morphological evaluation of bovine COC's before and after IVM

Comparing the groups, relative to the number of COC's matured, a increase in their maturation sign is observed, with 26.81% % (AR1), 21.67% (AR2) and 23.34% (AR3), respectively. Regardless of the treatment applied, the oocyte class is associated with their capacity to mature *in vitro* based on their morphological examination.

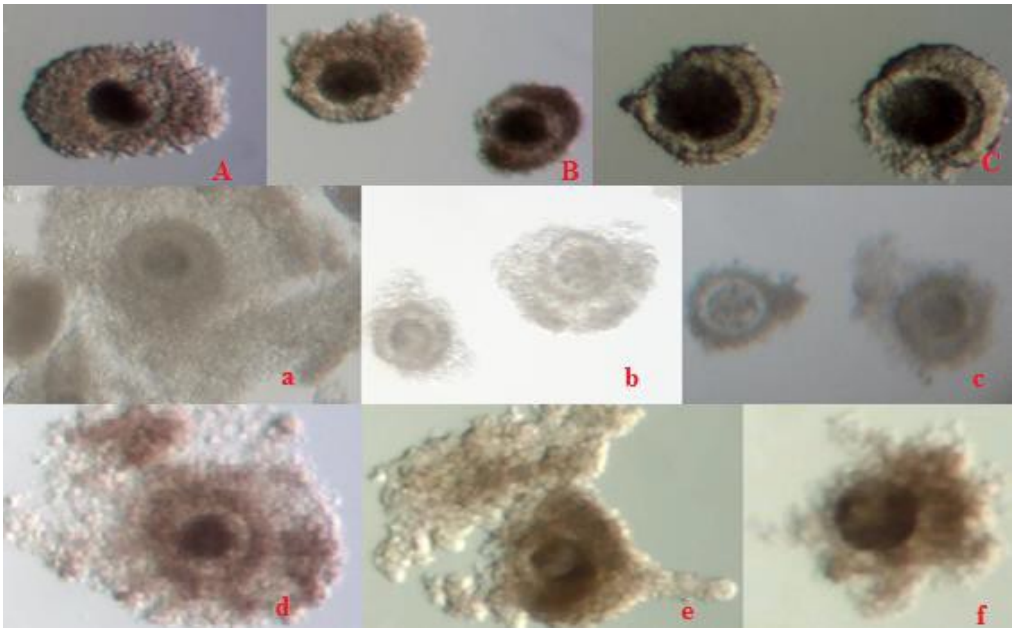


**Figure 2.** Aspects of COC's classification according to their morphological class and experimental group before IVM (A – I$^{st}$ class, B – II$^{nd}$ class, C – III$^{d}$ class), after IVM, group M (a, b, c), group AR (d, e, f)(5X)

These results are sustained also by BAX/BCL2 gene expression (unpublished data), where we observed that BCL-2 (anti-apoptotic gene) had higher levels in I$^{st}$ class COC's from rosmarinic acid (AR1) groups compared with the other groups and BAX (pro-apoptotic gene) level is indirectly proportional with the quality of the oocyte, with the highest level in III$^{d}$ class oocytes, what it means that both antioxidant supplementation and the quality of the oocyte has an important role in maintaining cellular viability.

Oxidative stress has negative effects on *in vitro* gametes and embryos (Agarwal et al., 2014; Beheshti et al, 2011) and excessive ROS production can't be controlled properly by the mammalian cells antioxidant systems (superoxide dismutase, glutathione system, thioredoxin system, catalase, thiol compounds) that scavenge ROS or prevents its formation due to the multiple potential sources of ROS, lack of physiological defense mechanisms etc (Sadeesh et al., 2014; Lu et al., 2013; Agarwal et al., 2014). That's why it's important to add antioxidants in media used in ART procedures (Sadeesh et al., 2014; Beheshti et al., 2011; Mircu et al., 2015; Sovernigo et al., 2017; Kang et al., 2016, Mbemya et al., 2017).

From literature data we know that rosmarinic acid antioxidant effects protects ovine spermatozoa during lyophilization by maintaining the sperm DNA integrity and after reconstitution of the freeze-dryed spermatozoa, they can sustain fertilization and even embryonic development (Olaciregui et al., 2017). Also in boar semen cryopreservation rosmarinic acid it is used as an antioxidant where improves the post-thaw quality of spermatozoa and the ability to fertilize (Malo et al., 2010; Luno et al., 2014; Luno et al., 2015).

Our preliminary results suggests that antioxidant properties of rosmarinic acid is effective also on bovine oocytes *in vitro* maturation. Further studies are needed to clarify the effects of rosmarinic acid used during IVM on further steps of IVF technique.

## Conclusions

- Supplementation of the cow's oocyte culture media with rosmarinic acid can determine a higher quantity of bovine oocytes matured *in vitro* based on morphological evaluation.
- Quality of the COC used for *in vitro* techniques has an important role in the success of the experiment.


## Aknowledgments

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